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Plasmid Chemokines and Colony-Stimulating Factors Enhance the Immunogenicity of DNA Priming-Viral Vector Boosting Human Immunodeficiency Virus Type 1 Vaccines

Dan H. Barouch,[1]* Paul F. McKay,[1] Shawn M. Sumida,[1] Sampa Santra,[1] Shawn S. Jackson,[1] Darci A. Gorgone,[1] Michelle A. Lifton,[1] Bimal K. Chakrabarti,[2] Ling Xu,[2] Gary J. Nabel,[2] and Norman L. Letvin[1]

Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215,[1] and Vaccine Research Center, National Institutes of Health, Bethesda, Maryland 20892[2]

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Heterologous "prime-boost" regimens that involve priming with plasmid DNA vaccines and boosting with recombinant viral vectors have been shown to elicit potent virus-specific cytotoxic T-lymphocyte responses. Increasing evidence, however, suggests that the utility of recombinant viral vectors in human populations will be significantly limited by preexisting antivector immunity. Here we demonstrate that the coadministration of plasmid chemokines and colony-stimulating factors with plasmid DNA vaccines markedly increases the immunogenicity of DNA prime-recombinant adenovirus serotype 5 (rAd5) boost and DNA prime-recombinant vaccinia virus (rVac) boost vaccine regimens in BALB/c mice. In mice with preexisting anti-Ad5 immunity, priming with the DNA vaccine alone followed by rAd5 boosting elicited only marginal immune responses. In contrast, cytokine-augmented DNA vaccine priming followed by rAd5 vector boosting was able to generate potent immune responses in mice with preexisting anti-Ad5 immunity. These data demonstrate that plasmid cytokines can markedly improve the immunogenicity of DNA prime-viral vector boost vaccine strategies and can partially compensate for antivector immunity.

Priming with plasmid DNA vaccines and boosting with recombinant viral vectors such as replication-defective adenoviruses and poxviruses have been shown to generate potent virus-specific cytotoxic T-lymphocyte (CTL) responses (2, 9, 11, 17, 19). Since virus-specific CTL responses are critical for the control of human immunodeficiency virus type 1 (HIV-1) (6, 10, 13, 15), these "prime-boost" strategies are being assessed as candidate AIDS vaccines (12). Recent studies have demonstrated that DNA prime-replication-defective adenovirus serotype 5 (rAd5) boost regimens (17) as well as DNA prime-recombinant-modified vaccinia virus Ankara (rMVA) boost regimens (2) afford significant control of pathogenic simian-human immunodeficiency virus (SHIV) challenges in rhesus monkeys. Similarly, DNA prime-rAd5 boost regimens have protected nonhuman primates against lethal challenge with Ebola virus (19).

The clinical utility of such prime-boost regimens, however, will likely be limited by preexisting immunity to the viral vector. Antivector immunity may result in the rapid elimination of the vaccine vector and thus could substantially reduce its immunogenicity. This problem is expected to be a major limitation for rAd5 vectors, since a large fraction of the human population has preexisting anti-Ad5 immunity as a result of natural exposure to Ad5. Strategies to improve these vaccine regimens are therefore needed.

One potential strategy for improving the immunogenicity of DNA prime-viral vector boost vaccine regimens is to improve the efficiency of the DNA prime by the coadministration of adjuvants. Our laboratory and others have previously demonstrated that plasmid cytokines can augment DNA vaccine-elicited humoral and cellular immune responses in both mice and rhesus monkeys (4–6, 16, 21, 23). Here we investigate the utility of chemoattractant plasmid cytokines in augmenting the immunogenicity of DNA prime-viral vector boost vaccine regimens in mice, both in the absence and in the presence of antivector immunity.

MATERIALS AND METHODS

Mice and immunizations. Six- to eight-week-old BALB/c mice were purchased from Charles River Laboratories (Wilmington, Mass.). For DNA immunizations using pVRC expression plasmids, 50 μg of pVRC-HIV-1 Env IIIB gp120 plasmid DNA vaccine (5) was first mixed with various amounts of sham plasmid or plasmid cytokines and then injected intramuscularly (i.m.) in 100 μl of sterile saline divided between the right and left quadriceps muscles. For rAd5 immunizations, mice were injected i.m. with various quantities of E1-deleted replication-incompetent rAd5-HIV-1 Env IIIB gp140ΔCFI in 100 μl of sterile phosphate-buffered saline (PBS). To prepare this rAd5 vector, a plasmid expressing the CXCR4-tropic HIV-1 HXB2 Env IIIB (GenBank accession no. K03455) was made synthetically by using codons typically found in human cells. To express a truncated mutant Env protein, a stop codon was introduced after position 680 to produce gp140. The Env protein was further changed by deleting amino acids 503 to 537 and 593 to 619, which removed the cleavage site sequence, the fusion domain, and a part of the spacer between the two heptad repeats to produce gp140ΔCFI. All mutations were confirmed by sequencing both strands of the cDNAs. Sequence analysis indicated that the codon-modified gp140ΔCFI was correct, except for the minor point substitutions previously described (7). The cDNAs were cloned into the *Xba*I and *Bam*HI sites of the Ad5 vector, which contained a deletion in E1 that rendered the vector replication-defective and a partial deletion or substitution in E3 that disrupted the coding sequences for the E3 proteins (14). The rAd5-gp140ΔCFI vaccine was then prepared as previously described (3, 19). For rVac immunizations, mice were injected intraperitoneally

* Corresponding author. Mailing address: Research East Rm. 113, Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, 330 Brookline Ave., Boston, MA 02215. Phone: (617) 667-4434. Fax: (617) 667-8210. E-mail: dbarouch@bidmc.harvard.edu.

(i.p.) in 100 μl of sterile PBS with various quantities of replication-competent vaccinia virus–HIV-1 Env IIIB gp160 (BH-10; Therion Biologics Corp., Cambridge, Mass.). To induce active anti-Ad5 immunity, mice were preimmunized i.m. with 5×10^9 particles of replication-defective Ad5 with no insert (ADV-ΔE1) in 100 μl of sterile PBS.

Tetramer staining. Tetrameric H-2D^d complexes folded around the HIV-1 IIIB V3 loop optimal P18 epitope peptide (P18-I10; RGPGRAFVTI) (20) were prepared and utilized to stain P18-specific CD8$^+$ T cells as described previously (1, 5). Mouse blood was collected in RPMI 1640 containing 40 U of heparin per ml. Following lysis of the erythrocytes (RBCs), 0.1 μg of phycoerythrin (PE)-labeled D^d/P18 tetramer in conjunction with allophycocyanin (APC)-labeled anti-CD8α monoclonal antibody (MAb) (Ly-2; Caltag, San Francisco, Calif.) was utilized to stain P18-specific CD8$^+$ T cells. The cells were washed in PBS containing 2% fetal bovine serum (FBS) and fixed in 0.5 ml of PBS containing 1.5% paraformaldehyde. Samples were analyzed by two-color flow cytometry on a FACS Calibur fluorescence-activated cell sorter (FACS) (Becton Dickinson Pharmingen, Mountain View, Calif.). Gated CD8$^+$ T lymphocytes were examined for staining with the D^d/P18 tetramer. CD8$^+$ T lymphocytes from naïve mice were utilized as negative controls and exhibited <0.1% tetramer staining.

Fresh ex vivo cytotoxicity assays. Cytotoxicity assays were utilized to measure functional CTL activity specific for the immunodominant D^d-restricted gp120 P18 epitope peptide (20) in freshly isolated, unstimulated splenocytes. Spleens were aseptically removed, and RBCs were removed from single-cell splenocyte suspensions using a hypotonic NH_4Cl-KCl lysis buffer. Splenocytes were then used directly as effector cells in standard ^{51}Cr-release cytotoxicity assays with P815 mastocytoma cells as target cells. P815 cells(10^6), pulsed overnight with 1 μg of P18 peptide per ml and 250 μCi of ^{51}Cr, were washed, and 10^4 target cells were added to various concentrations of effector cells in 200-μl reaction volumes. Spontaneous release and maximum release were measured by incubating target cells with media or 2% Triton X-100, respectively. After a 6-h incubation, 50 μl of supernatant was harvested, mixed with scintillation fluid, and assayed for radioactivity with a Wallac 1450 Microbeta liquid scintillation counter. Spontaneous release was <15% of maximum release. Percent specific lysis was calculated as: (experimental release − spontaneous release)/(maximum release − spontaneous release).

ELISPOT. Enzyme-linked immunospot (ELISPOT) assays were utilized to assess gamma interferon (IFN-γ) production by unfractionated splenocytes or splenocytes depleted of CD4$^+$ T cells or CD8$^+$ T cells. For depletion studies, splenocytes were incubated with magnetic microbeads coated with anti-CD4 (L3T4) or anti-CD8 (Ly-2) MAbs (Miltenyi Biotec, Auburn, Calif.), and separation was performed with MiniMACS columns according to the manufacturer's instructions. Cell depletions were approximately 95 to 98% efficient. IFN-γ secretion was then measured in response to the P18 epitope peptide or a pool of 47 overlapping 15-amino-acid peptides derived from HIV-1 IIIB Env gp120 (Centralised Facility for AIDS Reagents, Potters Bar, United Kingdom). Ninety-six-well multiscreen plates (Millipore, Bedford, Mass.) were coated overnight with 100 μl of 10-μg/ml rat anti-mouse IFN-γ (Pharmingen, San Diego, Calif.) per well in PBS and then washed with endotoxin-free Dulbecco's PBS (Life Technologies, Gaithersburg, Md.) containing 0.25% Tween 20 and blocked with PBS containing 5% FBS for 2 h at 37°C. The plates were washed three times with Dulbecco's PBS containing 0.25% Tween 20, rinsed with RPMI 1640 containing 10% FBS, and incubated in triplicate with 10^5 splenocytes per well in a 100-μl reaction volume containing 4 μg of peptide per ml. For studies utilizing the Env peptide pool, each peptide in the pool was present at 4 μg/ml. Following an 18-h incubation, the plates were washed nine times with Dulbecco's PBS containing 0.25% Tween 20 and once with distilled water. The plates were then incubated for 2 h with 75 μl of 5-μg/ml biotinylated rat anti-mouse IFN-γ (Pharmingen) per well, washed six times with Coulter Wash (Coulter Corporation, Miami, Fla.), and incubated for 2 h with a 1:500 dilution of streptavidin-alkaline phosphatase (Southern Biotechnology Associates, Birmingham, Ala.). Following five washes with Coulter Wash and one wash with PBS, the plates were developed with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) chromogen (Pierce, Rockford, Ill.), stopped by washing with tap water, air dried, and read with an ELISPOT reader (Hitech Instruments, Edgement, Pa.).

Cytokine secretion. A total of 4×10^6 splenocytes in 1 ml of RPMI 1640 containing 5% FBS were cultured with 0.1 μg of recombinant HIV-1 Env IIIB gp120 (Intracel, Cambridge, Mass.) per ml. After 72 h, supernatants were harvested and analyzed for the presence of cytokines by using commercial enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Cambridge, Mass.).

ELISAs. Serum anti-gp120 antibody titers from immunized mice were measured by a direct ELISA essentially as described previously (5). Ninety-six-well plates coated overnight with 100 μl of 1-μg/ml recombinant HIV-1 Env IIIB gp120 (Intracel, Cambridge, Mass.) per well in PBS were blocked for 2 h with PBS containing 2% bovine serum albumin (BSA) and 0.05% Tween 20. Sera were then added in serial dilutions and incubated for 1 h. The plates were washed three times with PBS containing 0.05% Tween 20 and incubated for 1 h with a 1:2,000 dilution of a peroxidase-conjugated affinity-purified rabbit anti-mouse secondary antibody (Jackson Laboratories, Bar Harbor, Maine). The plates were then washed three times, developed with TMB (KPL, Gaithersburg, Md.), stopped with 1% HCl, and analyzed at 450 nm with a Dynatech MR5000 ELISA plate reader. Serum anti-Ad5 antibody titers were measured by a similar direct ELISA in which plates were coated overnight with 10^8 ADV-ΔE1 particles per well.

Statistical analyses. Statistical analyses were performed with GraphPad Prism version 2.01 (GraphPad Software, Inc., 1996). Comparisons of cellular immune responses among groups of mice were performed by two-tailed *t* tests for two groups of animals or by analysis of variance (ANOVA) for more than two groups. Bonferroni adjustments were included when appropriate to account for multiple comparisons. In all cases, P values of <0.05 were considered significant.

RESULTS

Augmentation of DNA vaccine priming by plasmid GM-CSF and plasmid MIP-1α. We have demonstrated that the coadministration of plasmid granulocyte-macrophage colony stimulating factor (GM-CSF) and plasmid MIP-1α increased recruitment of dendritic cells to the site of inoculation and augmented DNA vaccine-elicited immune responses in mice (submitted). In the present study, we sought to determine whether DNA vaccines augmented by plasmid chemokines and GM-CSF would prime for improved immune responses following viral vector boost immunizations. Given the variability in the magnitude of immune responses observed in experimental animals vaccinated with DNA vaccines and certain plasmid cytokine adjuvants (4, 5, 16, 21), we first assessed the reproducibility of CD8$^+$ T-cell responses primed by GM-CSF/MIP-1α-augmented DNA vaccines in large groups of BALB/c mice ($n = 20$ per group). Mice were immunized with either 50 μg of gp120 DNA vaccine (5) plus 100 μg of sham plasmid or 50 μg of gp120 DNA vaccine plus 50 μg of plasmid murine GM-CSF and 50 μg of plasmid murine MIP-1α. Vaccine-elicited CD8$^+$ T-cell responses specific for the immunodominant H-2D^d-restricted P18 epitope (20) were assessed following immunization by measuring tetramer binding to CD8$^+$ T lymphocytes.

Mice primed with a single injection of the unadjuvanted gp120 DNA vaccine generated mean peak P18-specific responses of 1.14% of peripheral blood CD8$^+$ T lymphocytes on day 14 following primary immunization (Fig. 1). These responses declined to 0.34% by day 28 and were stably maintained thereafter. In contrast, mice primed with a single injection of the gp120/GM-CSF/MIP-1α DNA vaccine generated approximately eightfold-higher mean peak P18-specific responses of 8.76% of CD8$^+$ T lymphocytes several days earlier on day 10 following primary immunization. These responses declined to 1.03% on day 28 and were stably maintained thereafter. In both groups of mice, the standard errors were approximately 15% of the magnitude of the responses. Coadministration of plasmid GM-CSF and plasmid MIP-1α thus resulted in reproducible and robust increases in peak and memory P18-specific responses ($P < 0.0001$ comparing responses between groups on day 10 or day 49 by using two-tailed *t* tests).

Augmentation of DNA prime-viral vector boost regimens by plasmid cytokines. We next investigated the ability of rAd5 or rVac vectors expressing HIV-1 Env IIIB to boost cellular immune responses primed by the DNA vaccine with or without plasmid cytokine adjuvants. Mice primed with the gp120 DNA

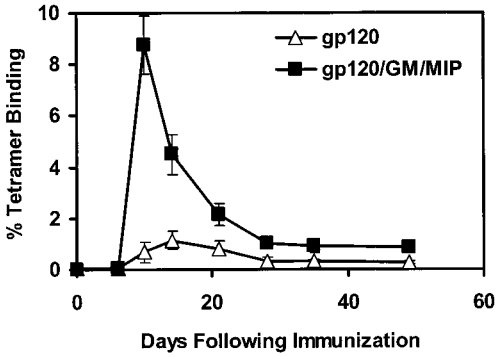


FIG. 1. Magnitude, kinetics, and variability of DNA vaccine-elicited $CD8^+$ T-lymphocyte responses. Groups of mice ($n = 20$ per group) were immunized once with either the gp120 DNA vaccine or the gp120/GM-CSF/MIP-1α DNA vaccine. Vaccine-elicited cellular immune responses were measured by tetramer binding to $CD8^+$ T lymphocytes. Means and standard errors of responses for each group are shown.

vaccine alone were boosted 3 months later with a dose titration of replication-defective rAd5-gp140ΔCFI (Fig. 2A) or replication-competent rVac-gp160 (Fig. 3A). Even at low doses, both vectors efficiently expanded P18-specific tetramer-positive $CD8^+$ T lymphocytes. A high dose of 10^9 particles of rAd5-gp140ΔCFI expanded P18-specific responses to 40 to 50% of peripheral blood $CD8^+$ T lymphocytes on day 7 following the boost, and these responses were stably maintained through day 21 (Fig. 2A). A low dose of 10^6 particles of rAd5-gp140ΔCFI

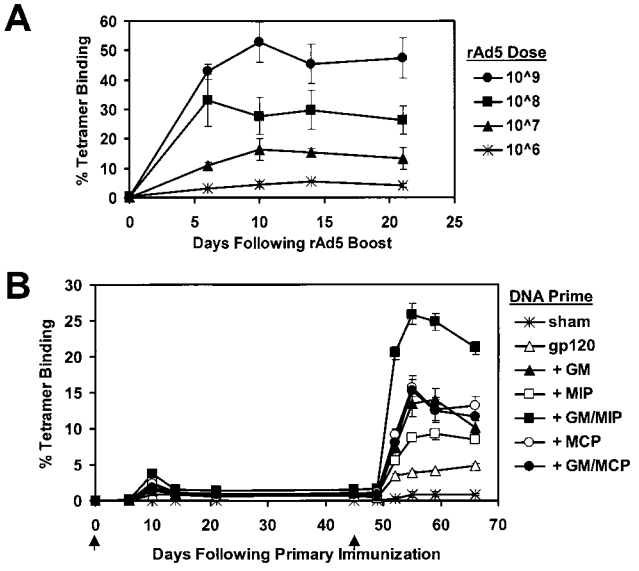


FIG. 2. Plasmid cytokines augment $CD8^+$ T-lymphocyte responses elicited by DNA prime-rAd5 boost regimens. (A) Groups of mice were primed with the gp120 DNA vaccine and then boosted with a dose titration of rAd5-gp140ΔCFI. (B) Groups of mice ($n = 4$ per group) were primed with sham plasmid, the gp120 DNA vaccine alone, or the gp120 DNA vaccine with the plasmid cytokine adjuvant GM-CSF, MIP-1α, GM-CSF plus MIP-1α, MCP-1, or GM-CSF plus MCP-1 and then boosted on day 45 with 10^6 particles of rAd5. Vaccine-elicited cellular immune responses were measured by tetramer binding to $CD8^+$ T lymphocytes. Arrows indicate immunizations.

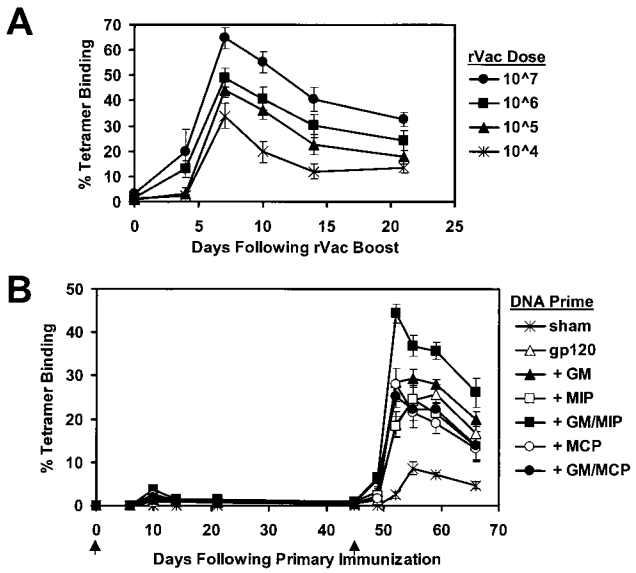
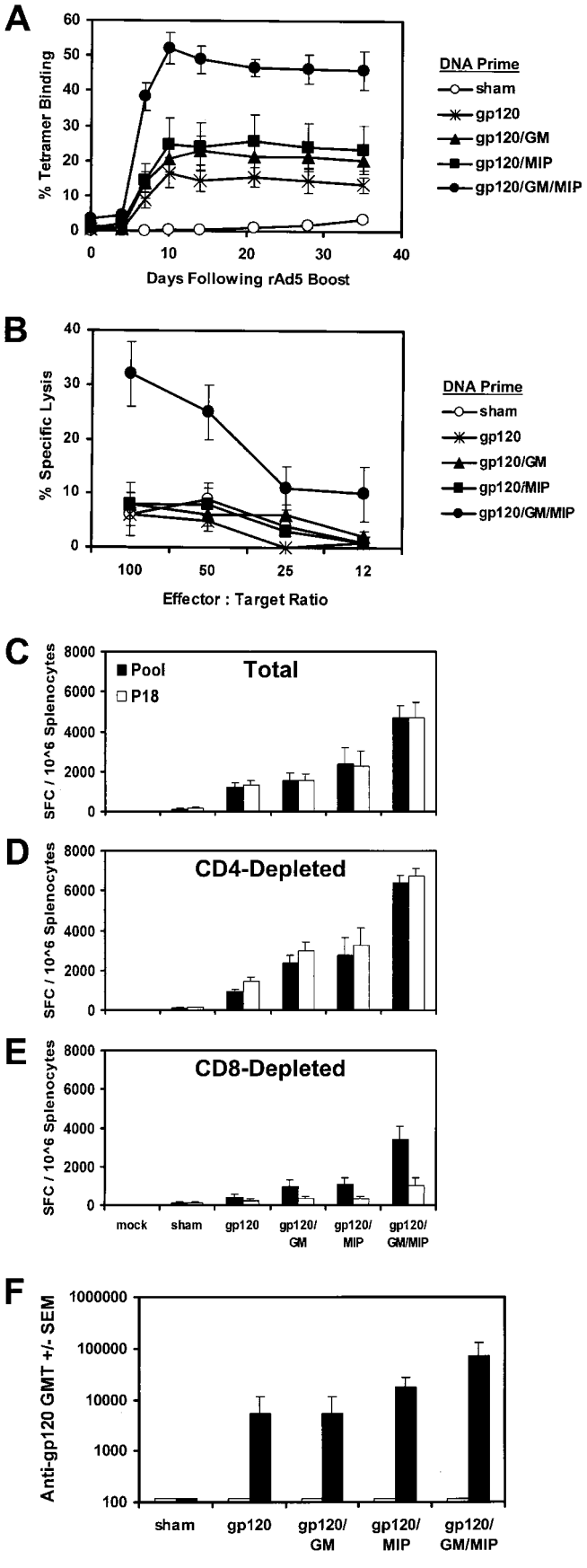


FIG. 3. Plasmid cytokines augment $CD8^+$ T-lymphocyte responses elicited by DNA prime-rVac boost regimens. (A) Groups of mice were primed with the gp120 DNA vaccine and then boosted with a dose titration of rVac-gp160. (B) Groups of mice ($n = 4$ per group) were primed with sham plasmid, the gp120 DNA vaccine alone, or the gp120 DNA vaccine with the plasmid cytokine adjuvant GM-CSF, MIP-1α, GM-CSF plus MIP-1α, MCP-1, or GM-CSF plus MCP-1 and then boosted on day 45 with 10^4 PFU of rVac-gp160. Vaccine-elicited cellular immune responses were measured by tetramer binding to $CD8^+$ T lymphocytes. Arrows indicate immunizations.

also boosted P18-specific responses to 4 to 5% of $CD8^+$ T lymphocytes. Different kinetics of expansion of $CD8^+$ T-cell responses were observed after boosting with rVac-gp160 vectors. P18-specific responses peaked on day 7 following the rVac boost and declined between days 7 and 21 (Fig. 3A). A low dose of 10^4 PFU of rVac-gp160 efficiently expanded P18-specific responses to 33% of $CD8^+$ T lymphocytes on day 7, and these responses declined to 12 to 14% of $CD8^+$ T lymphocytes by days 14 to 21.

We next assessed the ability of these rAd5 and rVac vectors to boost immune responses primed by cytokine-augmented DNA vaccines. Groups of mice ($n = 4$ per group) were primed on day 0 with sham plasmid, the gp120 DNA vaccine alone, or the gp120 DNA vaccine with the plasmid cytokines GM-CSF, MIP-1α, GM-CSF plus MIP-1α, MCP-1, or GM-CSF plus MCP-1 (Fig. 2B and 3B). Mice received 50 μg of each plasmid with sufficient sham plasmid to make the total inoculum 150 μg of DNA per animal. Primary cellular immune responses were highest in the animals that received the gp120/GM-CSF/MIP-1α DNA vaccine.

These animals were boosted with low doses of rAd5-gp140ΔCFI (10^6 particles) or rVac-gp160 (10^4 PFU) on day 45 following primary immunization. The rAd5-gp140ΔCFI boost resulted in an efficient expansion of the DNA-primed memory responses in all animals (Fig. 2B). Interestingly, the mice that received the gp120/GM-CSF/MIP-1α DNA vaccine prime maintained the highest responses following the boost. In fact, the relative hierarchy of responses among groups of mice that received the different priming regimens was unchanged by the



rAd5 boost. In mice primed with the gp120 DNA vaccine alone, P18-specific responses expanded to 4 to 5% of $CD8^+$ T lymphocytes following the rAd5-gp140ΔCFI boost. In mice primed with the gp120/GM-CSF/MIP-1α DNA vaccine, responses expanded to 20 to 25% of $CD8^+$ T lymphocytes. Intermediate responses were observed in the groups that received the other plasmid cytokine adjuvants. These data demonstrate that the augmentation of DNA vaccine priming by a variety of plasmid cytokine adjuvants resulted in increased efficiency of the rAd5 boost. Similarly, mice primed with the gp120/GM-CSF/MIP-1α DNA vaccine developed higher responses than mice primed with the gp120 DNA vaccine alone following the rVac-gp160 boost (Fig. 3B). However, differences among groups were less clear following the rVac boost than following the rAd5 boost.

Functional analysis of vaccine-elicited immune responses. We next sought to investigate the functional characteristics of the immune responses elicited by the cytokine-augmented DNA prime-rAd5 boost regimens. Groups of mice ($n = 4$ per group) were primed with sham plasmid, the gp120 DNA vaccine alone, or the gp120 DNA vaccine with the plasmid cytokine adjuvant GM-CSF, MIP-1α, or GM-CSF plus MIP-1α. Mice received 50 μg of each plasmid with sufficient sham plasmid to make the total inoculum 150 μg of DNA. With the exception of the sham-vaccinated mice, all groups were boosted with 50 μg of gp120 DNA vaccine alone at week 8. All groups then received a third injection of 10^6 particles of rAd5-gp140ΔCFI at week 24. The rAd5 boost efficiently expanded P18-specific tetramer-positive $CD8^+$ T lymphocytes in all groups, and the hierarchy of responses among groups was preserved (Fig. 4A). Trends toward higher responses following the rAd5 boost were observed in mice primed with the gp120/GM-CSF DNA vaccine or the gp120/MIP-1α DNA vaccine than in mice primed with the gp120 DNA vaccine alone. In contrast, significantly higher responses were elicited in mice primed with the gp120/GM-CSF/MIP-1α DNA vaccine than in mice primed with the gp120/MIP-1α DNA vaccine ($P < 0.01$), the gp120/GM-CSF DNA vaccine ($P < 0.01$), or the gp120 DNA vaccine alone ($P < 0.001$ comparing responses among groups on day 14 following the rAd5-gp140ΔCFI boost by ANOVA with Bonferroni adjustments to account for multiple comparisons). Remarkably potent and durable P18-specific responses of 45 to 52% of $CD8^+$ T lymphocytes were elicited in mice primed with the gp120/GM-CSF/MIP-1α DNA vaccine

FIG. 4. Functional analysis of vaccine-elicited immune responses. Groups of mice ($n = 4$ per group) were primed with sham plasmid, the gp120 DNA vaccine alone, or the gp120 DNA vaccine with the plasmid cytokine adjuvant GM-CSF, MIP-1α, or GM-CSF plus MIP-1α. These mice were then boosted at week 8 with the gp120 DNA vaccine alone and at week 24 with 10^6 particles of rAd5-gp140ΔCFI. Vaccine-elicited cellular immune responses were assessed by (A) tetramer binding to $CD8^+$ T lymphocytes, (B) fresh ex vivo cytotoxicity assays, and pooled peptide and P18-specific IFN-γ ELISPOT assays using either unfractionated (C), CD4-depleted (D), or CD8-depleted (E) splenocytes. SFC, spot-forming cells. (F) Vaccine-elicited humoral immune responses were assessed by a gp120 ELISA prior to immunization (white bars) and 28 days after the rAd5-gp140ΔCFI boost (black bars).

TABLE 1. Cytokine secretion profiles of antigen-stimulated splenocytes[a]

Stimulation	Level of cytokine secretion (pg/ml)				
	IFN-γ	IL-2	IL-4	IL-10	TNF-α
Mock	2,800 ± 932	1,887 ± 381	66 ± 17	263 ± 9	29 ± 0
Sham	4,399 ± 729	2,487 ± 450	109 ± 9	284 ± 10	34 ± 2
gp120	172,144 ± 50,77	32,793 ± 6,459	515 ± 72	570 ± 81	34 ± 3
gp120/GM	183,127 ± 41,181	38,983 ± 2,396	503 ± 72	771 ± 21	37 ± 1
gp120/MIP	286,344 ± 62,971	45,862 ± 4,800	513 ± 43	610 ± 49	37 ± 3
gp120/GM/MIP	1,858,355 ± 500,472	199,279 ± 43,340	306 ± 66	813 ± 91	39 ± 1

[a]Groups of mice ($n = 4$ per group) were primed with a sham plasmid, the gp120 DNA vaccine alone, or cytokine-augmented DNA vaccines. These mice were then boosted at week 8 with DNA and at week 24 with 10^6 particles of rAd5. Levels of cytokine secretion (picograms per milliliter) by recombinant gp120-stimulated splenocytes were assessed by ELISA. Splenocytes from naïve mice (mock) were included as a control to determine baseline levels of cytokine secretion.

and boosted with the gp120 DNA vaccine and low-dose rAd5-gp140ΔCFI.

In order to determine whether these high-frequency $CD8^+$ T-lymphocyte responses in fact represented functional CTLs, these mice were sacrificed on day 42 following the rAd5 boost. Splenocytes were isolated and evaluated in fresh ex vivo cytotoxicity assays. Unstimulated splenocytes of mice primed with the gp120/GM-CSF/MIP-1α DNA vaccine and boosted with rAd5 exhibited potent fresh cytotoxicity (Fig. 4B). IFN-γ secretion by splenocytes of these mice was also assessed by ELISPOT assays using the Env IIIB peptide pool or the P18 epitope peptide. Trends toward higher ELISPOT responses were observed in mice primed with the gp120/GM-CSF DNA vaccine or the gp120/MIP-1α DNA vaccine as compared to those in mice primed with the gp120 DNA vaccine alone (Fig. 4C). In contrast, splenocytes of mice primed with the gp120/GM-CSF/MIP-1α DNA vaccine exhibited high-frequency ELISPOT responses of over 5,000 spot-forming cells per 10^6 splenocytes following exposure to pooled peptides or the P18 peptide. These responses were significantly higher than the responses observed in mice primed with the gp120/MIP-1α DNA vaccine ($P < 0.05$), the gp120/GM-CSF DNA vaccine ($P < 0.01$), or the gp120 DNA vaccine alone ($P < 0.01$ comparing ELISPOT responses among groups by ANOVA with Bonferroni adjustments to account for multiple comparisons). Similar results were also observed in ELISPOT assays using splenocytes depleted of $CD4^+$ T lymphocytes or $CD8^+$ T lymphocytes (Fig. 4D and E).

Table 1 shows cytokine secretion profiles of antigen-stimulated splenocytes from these groups of mice. Remarkably high levels of IFN-γ and interleukin-2 (IL-2) secretion were observed in the mice primed with cytokine-augmented DNA vaccines, particularly the animals that received the gp120/GM-CSF/MIP-1α DNA vaccine. Moreover, following the rAd5-gp140ΔCFI boost, 10-fold-higher anti-gp120 antibody titers were elicited in mice that were primed with the gp120/GM-CSF/MIP-1α DNA vaccine than in mice that were primed with the gp120 DNA vaccine alone following the rAd5-gp140ΔCFI boost (Fig. 4F). These data demonstrate that coadministration of these plasmid cytokines with DNA vaccine priming resulted in a marked enhancement of vaccine-elicited humoral and cellular immune responses following the rAd5 boost.

Inhibitory effects of preexisting anti-Ad5 immunity. An important practical limitation of rAd5 vectors is that a large percentage of the human population has preexisting immunity to Ad5 that may blunt the immunogenicity of rAd5 vectors. To model this phenomenon, we evaluated the effects of preexisting anti-Ad5 immunity on immune responses elicited by DNA prime-rAd5 boost vaccine regimens in mice. Groups of mice ($n = 4$ per group) were preimmunized 28 days prior to vaccination with either saline or empty Ad5 (ADV-ΔE1 [5×10^9 particles]), to induce active anti-Ad5 immunity. Mice preimmunized with saline and primed with the gp120 DNA vaccine alone exhibited potent expansions of tetramer-positive $CD8^+$ T-cell responses following the rAd5-gp140ΔCFI boost as expected (Fig. 5A). In these groups of animals, P18-specific responses expanded to 25 to 30% of $CD8^+$ T lymphocytes following a boost with 10^9 particles of rAd5-gp140ΔCFI and to 3 to 5% of $CD8^+$ T lymphocytes following a boost with 10^6 particles of rAd5-gp140ΔCFI. In contrast, mice preimmunized with empty Ad5 and similarly primed with the gp120 DNA vaccine alone exhibited a dramatic blunting of tetramer-positive $CD8^+$ T-cell responses following the rAd5-gp140ΔCFI boost (Fig. 5B). P18-specific responses expanded to only 3 to 5% of $CD8^+$ T lymphocytes following a boost with 10^9 particles of rAd5-gp140ΔCFI, and minimal boosting effects were observed with lower doses. Thus, preexisting anti-Ad5 immunity induced by this regimen resulted in a 3-log reduction of the effective rAd5-gp140ΔCFI dose and a 90% reduction in the absolute response. Anti-Ad5 antibody titers in mice preimmunized with Ad5 were confirmed by ELISA (Fig. 5D).

We reasoned that mice that received a more potent DNA vaccine priming regimen may be capable of overcoming in part the inhibitory effects of preexisting anti-Ad5 immunity. Mice preimmunized with empty Ad5 and primed with the gp120/GM-CSF/MIP-1α DNA vaccine exhibited a partial recovery of tetramer-positive $CD8^+$ T-cell responses following rAd5-gp140ΔCFI boost. P18-specific responses expanded to 12 to 15% of $CD8^+$ T lymphocytes following a boost with 10^9 particles of rAd5 and to 3 to 5% of $CD8^+$ T lymphocytes following a boost with 10^7 particles of rAd5. Thus, the coadministration of these plasmid cytokine adjuvants with DNA vaccine priming restored approximately 2 of the 3 logs of the effective rAd5 dose and 50% of the absolute response. Partial restoration of gp120-specific antibody responses was also evident following the rAd5 boosts in Ad5 preimmunized mice that were primed with the gp120/GM-CSF/MIP-1α DNA vaccine compared to those given the gp120 DNA vaccine alone (Fig. 5E).

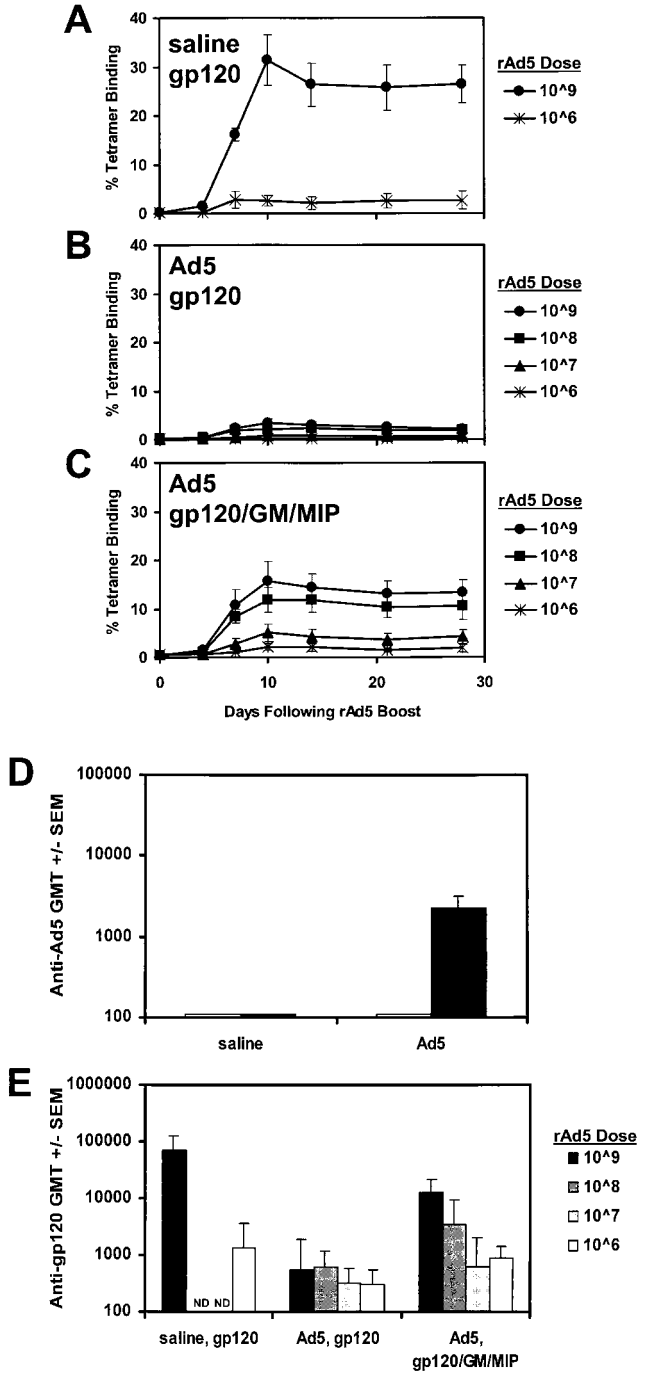


FIG. 5. Preexisting anti-Ad5 immunity suppresses DNA/rAd5 immune responses. Groups of mice ($n = 4$ per group) were preimmunized with either saline (A) or 5×10^9 particles of empty Ad5 (B and C) to induce anti-Ad5 immunity. Mice were primed with the gp120 DNA vaccine (A and B) or the gp120/GM-CSF/MIP-1α DNA vaccine (C) and then boosted with various doses of rAd5-gp140ΔCFI. Vaccine-elicited cellular immune responses were measured by tetramer binding to $CD8^+$ T lymphocytes following the rAd5 boost. (D) Anti-Ad5 antibody responses were measured by ELISA prior to (white bars) and 28 days after (black bars) preimmunization with saline or Ad5. (E) Vaccine-elicited humoral immune responses were assessed by a gp120 ELISA. ND, not done.

DISCUSSION

Plasmid DNA prime-viral vector boost vaccine regimens have been shown to elicit potent virus-specific cellular immune responses in animal models and are therefore being developed as candidate vaccines for HIV-1 and other pathogens (2, 9, 11, 17, 19). These prime-boost strategies, however, may be considerably less immunogenic in humans than in animal models, particularly in individuals with preexisting immunity to the viral vector. Strategies to improve these vaccine regimens are therefore needed. Here we show that coadministration of plasmid cytokines that improved the efficiency of DNA vaccine priming also resulted in potent and durable augmentation of the overall immunogenicity of DNA prime-viral vector boost vaccine regimens in BALB/c mice. Moreover, priming with cytokine-augmented DNA vaccines was more effective than priming with DNA vaccines alone in overcoming the inhibitory effects of preexisting anti-Ad5 immunity following rAd5-gp140ΔCFI boosts.

These results demonstrate that the efficiency of DNA vaccine priming is an important factor in determining the magnitude of immune responses following viral vector boosts. It is likely that mice primed with cytokine-augmented DNA vaccines were more effectively boosted with rAd5 than mice primed with DNA vaccines alone because they had larger numbers of circulating antigen-specific memory T lymphocytes available to expand following a second exposure to antigen. We cannot exclude the possibility that the plasmid cytokines may have also directly altered or improved the function of the vaccine-elicited T lymphocytes. However, we believe this to be unlikely, since we were unable to detect differences in the immunophenotypes of the tetramer-positive $CD8^+$ T lymphocytes elicited by the cytokine-augmented DNA vaccines as compared with the DNA vaccines alone (data not shown). Moreover, the rAd5 vector boost expanded the DNA-primed memory tetramer-positive $CD8^+$ T-lymphocyte responses in a linear fashion and did not alter the relative hierarchies of responses among groups of DNA-primed mice (Fig. 2B and 4A). Interestingly, the differences in responses among groups of mice were less well maintained after the rVac boost than after the rAd5 vector boost, perhaps as a result of the more complex kinetics of the replication-competent rVac vector compared to that of the replication-incompetent rAd5 vector (Fig. 2A and 3A).

Preexisting anti-Ad5 immunity in human populations may limit the utility of the DNA prime-rAd5 boost vaccines under development. The results of this study show that cytokine-augmented DNA vaccine priming is more effective than DNA vaccine priming alone (24) as a strategy to overcome the inhibitory effects of preexisting antivector immunity. We investigated the hypothesis that a larger pool of antigen-specific memory lymphocytes induced by cytokine-augmented DNA vaccine priming as compared with DNA vaccine priming alone may require a lower "threshold dose" of rAd5 to achieve a defined boosting effect. The threshold dose of rAd5 necessary to elicit a response of 3 to 5% of tetramer-positive $CD8^+$ T lymphocytes following the boost was 10^6 particles of rAd5 in mice primed with the gp120 DNA vaccine alone in the absence of preexisting anti-Ad5 immunity (Fig. 5A). In similarly primed mice in the presence of preexisting immunity, the threshold

dose required to achieve this response was increased to 10^9 particles of rAd5 (Fig. 5B). In mice with preexisting immunity that were primed with the gp120/GM-CSF/MIP-1α DNA vaccine, however, this threshold dose was reduced to 10^7 particles of rAd5 (Fig. 5C). These data demonstrate that increasing the efficiency of DNA vaccine priming using plasmid cytokine adjuvants effectively lowered the threshold dose of rAd5 vector required to achieve this defined boosting effect, thus partially overcoming the suppressive effects of preexisting anti-Ad5 immunity.

These data suggest that priming with cytokine-augmented DNA vaccines and boosting with a viral vector warrants further investigation as a potential candidate HIV-1 vaccine strategy. Augmenting the efficiency of DNA vaccine priming may be particularly important in the setting of preexisting antivector immunity. Other strategies are also being investigated to mitigate the effects of preexisting antivector immune responses on the immunogenicity of rAd vectors, including developing vectors from rare adenovirus serotypes (18), utilizing adenoviruses isolated from nonhuman primates rather than humans (22), and constructing recombinant vectors that specifically evade existing immune responses (8). It is possible that cytokine-augmented DNA vaccine priming could ultimately be combined with some of these second-generation vectors to develop further improved DNA prime-viral vector boost vaccine regimens.

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REFERENCES

1. **Altman, J. D., P. A. H. Moss, P. J. R. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis.** 1996. Phenotypic analysis of antigen-specific T lymphocytes. Science **274:**94–96.
2. **Amara, R. R., F. Villinger, J. D. Altman, S. L. Lydy, S. P. O'Neil, S. I. Staprans, D. C. Montefiori, Y. Xu, J. G. Herndon, L. S. Wyatt, M. A. Candido, N. L. Kozyr, P. L. Earl, J. M. Smith, H.-K. Ma, B. D. Grimm, M. L. Hulsey, H. M. McClure, J. M. McNicholl, B. Moss, and H. L. Robinson.** 2001. Control of a mucosal challenge and prevention of clinical AIDS in rhesus monkeys by a multiprotein DNA/MVA vaccine. Science **292:**69–74.
3. **Aoki, K., C. Barker, X. Danthinne, M. J. Imperiale, and G. J. Nabel.** 1999. Efficient generation of recombinant adenoviral vectors by Cre-lox recombination in vitro. Mol. Med. **5:**224–231.
4. **Barouch, D. H., S. Santra, T. D. Steenbeke, X. X. Zheng, H. C. Perry, M.-E. Davies, D. C. Freed, A. Craiu, T. B. Strom, J. W. Shiver, and N. L. Letvin.** 1998. Augmentation and suppression of immune responses to an HIV-1 DNA vaccine by plasmid cytokine/Ig administration. J. Immunol. **161:**1875–1882.
5. **Barouch, D. H., S. Santra, K. Tenner-Racz, P. Racz, M. J. Kuroda, J. E. Schmitz, S. S. Jackson, M. A. Lifton, D. C. Freed, H. C. Perry, M. E. Davies, J. W. Shiver, and N. L. Letvin.** 2002. Potent CD4+ T cell responses elicited by a bicistronic HIV-1 DNA vaccine expressing gp120 and GM-CSF. J. Immunol. **168:**562–568.
6. **Barouch, D. H., S. Santra, J. E. Schmitz, M. J. Kuroda, T.-M. Fu, W. Wagner, M. Bilska, A. Craiu, X. X. Zheng, G. R. Krivulka, K. Beaudry, M. A. Lifton, C. E. Nickerson, W. L. Trigona, K. Punt, D. C. Freed, L. Guan, S. Dubey, D. Casimiro, A. Simon, M.-E. Davies, M. Chastain, T. B. Strom, R. S. Gelman, D. C. Montefiori, M. G. Lewis, E. A. Emini, J. W. Shiver, and N. L. Letvin.** 2000. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. Science **290:**486–492.
7. **Chakrabarti, B. K., W.-P. Kong, B.-Y. Wu, Z.-Y. Yang, J. Friborg, X. Ling, S. R. King, D. C. Montefiori, and G. J. Nabel.** 2002. Modifications of the human immunodeficiency virus envelope glycoprotein enhance immunogenicity for genetic immunization. J. Virol. **76:**5357–5368.
8. **Gall, J. G. D., R. G. Crystal, and E. Falck-Pedersen.** 1998. Construction and characterization of hexon-chimeric adenoviruses: specification of adenovirus serotype. J. Virol. **72:**10260–10264.
9. **Hanke, T., R. V. Samuel, T. J. Blanchard, V. C. Neumann, T. M. Allen, J. E. Boyson, S. A. Sharpe, N. Cook, G. L. Smith, D. I. Watkins, M. P. Cranage, and A. J. McMichael.** 1999. Effective induction of simian immunodeficiency virus-specific cytotoxic T lymphocytes in macaques by using a multiepitope gene and DNA prime-modified vaccinia virus Ankara boost vaccination regimen. J. Virol. **73:**7524–7532.
10. **Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safrit, J. Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson, and D. D. Ho.** 1999. Dramatic rise in plasma viremia after CD8+ T cell depletion in simian immunodeficiency virus-infected macaques. J. Exp. Med. **189:**991–998.
11. **Kent, S. J., A. Zhao, S. J. Best, J. D. Chandler, D. B. Boyle, and I. A. Ramshaw.** 1998. Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. J. Virol. **72:**10180–10188.
12. **Letvin, N. L., D. H. Barouch, and D. C. Montefiori.** 2002. Prospects for vaccine protection against HIV-1 infection and AIDS. Annu. Rev. Immunol. **20:**73–99.
13. **Musey, L., J. Hughes, T. Schacker, T. Shea, L. Corey, and M. J. McElrath.** 1997. Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. N. Engl. J. Med. **337:**1267–1274.
14. **Ohno, T., D. Gordon, H. San, V. J. Pompili, M. J. Imperiale, G. J. Nabel, and E. G. Nabel.** 1994. Gene therapy for vascular smooth muscle cell proliferation after arterial injury. Science **265:**781–784.
15. **Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann.** 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science **283:**857–860.
16. **Sedegah, M., W. Weiss, J. B. Sacci, Jr., Y. Charoenvit, R. Hedstrom, K. Gowda, V. F. Majam, J. Tine, S. Kumar, P. Hobart, and S. L. Hoffman.** 2000. Improving protective immunity induced by DNA-based immunization: priming with antigen and GM-CSF-encoding plasmid DNA and boosting with antigen-expressing recombinant poxvirus. J. Immunol. **164:**5905–5912.
17. **Shiver, J. W., T. M. Fu, L. Chen, D. R. Casimiro, M. E. Davies, R. K. Evans, Z. Q. Zhang, A. J. Simon, W. L. Trigona, S. A. Dubey, L. Huang, V. A. Harris, R. S. Long, X. Liang, L. Handt, W. A. Schleif, L. Zhu, D. C. Freed, N. V. Persaud, L. Guan, K. S. Punt, A. Tang, M. Chen, K. A. Wilson, K. B. Collins, G. J. Heidecker, V. R. Fernandez, H. C. Perry, J. G. Joyce, K. M. Grimm, J. C. Cook, P. M. Keller, D. S. Kresock, H. Mach, R. D. Troutman, L. A. Isopi, D. M. Williams, Z. Xu, K. E. Bohannon, D. B. Volkin, D. C. Montefiori, A. Miura, G. R. Krivulka, M. A. Lifton, M. J. Kuroda, J. E. Schmitz, N. L. Letvin, M. J. Caulfield, A. J. Bett, R. Youil, D. C. Kaslow, and E. A. Emini.** 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature **415:**331–335.
18. **Smith, C. A., L. S. Woodruff, C. Rooney, and G. R. Kitchingman.** 1998. Extensive cross-reactivity of adenovirus-specific cytotoxic T cells. Hum. Gene Ther. **9:**1419–1427.
19. **Sullivan, N. J., A. Sanchez, P. E. Rollin, Z. Y. Yang, and G. J. Nabel.** 2000. Development of a preventive vaccine for Ebola virus infection in primates. Nature **30:**605–609.
20. **Takahashi, H., Y. Nakagawa, C. D. Pendleton, R. A. Houghten, K. Yokomuro, R. N. Germain, and J. A. Berzofsky.** 1992. Induction of broadly cross-reactive cytotoxic T cells recognizing an HIV-1 envelope determinant. Science **255:**333–336.
21. **Weiss, W. R., K. J. Ishii, R. C. Hedstrom, M. Sedegah, M. Ichino, K. Barnhart, D. M. Klinman, and S. L. Hoffman.** 1998. A plasmid encoding murine granulocyte-macrophage colony-stimulating factor increases protection conferred by a malaria DNA vaccine. J. Immunol. **161:**2325–2332.
22. **Xiang, Z., G. Gao, A. Reyes-Sandoval, C. J. Cohen, Y. Li, J. M. Bergelson, J. M. Wilson, and H. C. J. Ertl.** 2002. Novel, chimpanzee serotype 68-based adenoviral vaccine carrier for induction of antibodies to a transgene product. J. Virol. **76:**2667–2675.
23. **Xiang, Z., and H. C. J. Ertl.** 1995. Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. Immunity **2:**129–135.
24. **Yang, Z.-Y., L. S. Wyatt, W.-P. Kong, Z. Moodie, B. Moss, and G. J. Nabel.** 2003. Overcoming immunity to a viral vaccine by DNA priming before vector boosting. J. Virol. **77:**799–803.